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## On the relevance of carnosine and carnosinase for the development of diabetic nephropathy

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# CHAPTER 4

## **DIFFERENT CONFORMATIONAL FORMS OF SERUM CARNOSINASE DETECTED BY A NEWLY DEVELOPED SANDWICH ELISA FOR THE MEASUREMENTS OF CARNOSINASE CONCENTRATIONS**

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### ABSTRACT

Serum carnosinase (CN-1) measurements are at present mainly performed by assessing enzyme activity. This method is time consuming, not well suited for large series of samples and can be discordant to measurements of CN-1 protein concentrations. To overcome these limitations we developed sandwich ELISA assays using different anti-CN-1 antibodies, i.e. ATLAS (polyclonal IgG) and RYSK173 (monoclonal IgG1). With the ATLAS-based assay, similar amounts of CN-1 were detected in serum and both EDTA and heparin plasma. The RYSK173-based assay detected CN-1 in serum in all individuals at significant lower concentrations compared to the ATLAS-based assay (range: 0.1- 1.8 vs. 1 – 50 µg/ml, RYSK- vs. ATLAS-based,  $P < 0.01$ ). CN-1 detection with the RYSK-based assay was increased in EDTA plasma, albeit at significantly lower concentrations compared to ATLAS. In heparin plasma CN-1 was also poorly detected with the RYSK-based assay. Addition of DTT to serum increased the detection of CN-1 in the RYSK-based assay almost to the levels found in the ATLAS-based assay. Both ELISA assays were highly reproducible ( $R: 0.99$ ,  $P < 0.01$  and  $R: 0.93$ ,  $P < 0.01$ , for the RYSK- and ATLAS-based assays, respectively). Results of the ATLAS-based assay showed a positive correlation with CN-1 activity ( $R: 0.59$ ,  $P < 0.01$ ), while this was not the case for the RYSK-based assay. However, there was a negative correlation between CN-1 activity and the proportion of CN-1 detected in the RYSK-based assay, i.e. CN-1 detected with the RYSK-based assay / CN-1 detected with the ATLAS-based assay  $\times 100\%$  ( $R: -0.51$ ,  $P < 0.01$ ), suggesting that the RYSK-based assay most likely detects a CN-1 conformation with low CN-1 activity. RYSK173, and ATLAS antibodies reacted similar in western blot, irrespective of PNGase treatment. Binding of RYSK173 in serum was not due to differential N-glycosylation as demonstrated by mutant CN-1 cDNA constructs. In conclusion our study demonstrates a good correlation between enzyme activity and CN-1 protein concentration in ELISA and suggests the presence of different CN-1 conformations in serum. The relevance of these different conformations is still elusive and needs to be addressed in further studies.

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## INTRODUCTION

Carnosine-like peptides, i.e. L-carnosine ( $\beta$ -alanyl-L-histidine), homocarnosine ( $\gamma$ -aminobutyryl-L-histidine) and anserine ( $\beta$ -alanyl-L-1-methylhistidine), are a family of histidine derivatives that are present in a wide variety of vertebrate tissues, e.g. skeletal muscles and brain (62). Elevated carnosine concentrations in human skeletal muscle lead to improved muscle performance in high-intensity exercise in both untrained and trained individuals (160, 161). This suggests an important role of the dipeptide in the homeostasis of contracting muscle cells during anaerobic energy delivery, most likely by acting as physiological pH buffer (162, 163). Apart from muscle performance improving properties (160-163), L-carnosine seems to be implicated in neuroprotection (164) and seems to play a role in the development of the olfactory system and organization of hypothalamic neuronal networks (165).

Plasma carnosine concentrations vary significantly over a 24 hrs period, depending on intestinal absorption of intact carnosine (68, 70), muscle exercise (166) and activity of the carnosine synthetase (167). In addition, carnosine concentrations in plasma and tissue depend on the activities of intra- and extracellular dipeptidases known as carnosinases.

Carnosinases, first isolated from the porcine kidney (45), are widely distributed in tissues of rodents and higher mammals (45, 48, 50, 167). Two types of carnosinases have been identified in humans encoded by the *CNDP1* and *CNDP2* genes (55). The gene products are also known as serum carnosinase (CN-1) and non-specific carnosine dipeptidase (CN-2), respectively. While CN-1 was identified as a dipeptidase that specifically hydrolyzes carnosine and homocarnosine, CN-2 has a broader specificity than CN-1 and is not able to hydrolyze homocarnosine and sensitive to inhibition by bestatin (55).

We have recently demonstrated that susceptibility to diabetic nephropathy (DN) is strongly associated with a (CTG)<sub>n</sub> polymorphism in the *CNDP1* gene (44), affecting serum carnosinase secretion (168). Diabetic patients homozygous for (CTG)<sub>5</sub> have a lower risk to develop diabetic nephropathy and have a lower plasma CN-1 activity (44). Interestingly, over-expression of *CNDP1* in the Db/Db model results in early onset of diabetes, while in Db/Db mice fed with carnosine the onset of diabetes is retarded (113).

Currently, carnosinase measurements are mostly performed by measuring enzyme activity (44, 54, 55, 59). Yet, CN-1 activity can be modulated by the presence of homocarnosine (169), binding of metal ions in the active centre (54) and by N-glycosylation of CN-1 (170). Hence serum CN-1 activities can be discordant to CN-1 protein concentrations. In order to assess whether a low serum CN-1 activity is due to a low CN-1 concentration, estimation of

the amount of CN-1 in serum is required. Although, this could be performed by western blotting in a semi-quantitative fashion, this method is time consuming and therefore is not well suited for routine testing of numerous samples. The present study was therefore conducted to set-up ELISA assays that would allow the measurement of CN-1 in large series of individuals.

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## RESEARCH DESIGN AND METHODS

### *Cell culture and transfection*

Cos-7 cells (Invitrogen, Karlsruhe, Germany) were cultured in Dulbeccos Modified Eagle Medium (DMEM, PAA The Cell Culture Company, Pasching, Austria) enriched with 10% FCS and 1% Penicillin/ Streptomycin at 37°C and 5% CO<sub>2</sub>. The cells were co-transfected with the various CNDP1 constructs and pCruz-GFP<sub>TM</sub> (Santa Cruz, Heidelberg, Germany) by Nanofectin according to the manufacturer's instructions (PAA The Cell Culture Company, Pasching, Austria). Four hours later, medium was replaced by DMEM medium containing 1% Penicillin/ Streptomycin. Cells and supernatants were analyzed 48 hours later. Transfection efficiency was assessed on an aliquot of the cell suspension by FACS analysis using green fluorescent protein (GFP) as read-out. In general transfection efficiency was above 70%. In all experiments supernatants were concentrated using a Centricon Centrifugal Filter device 30.000 MW (Millipore, Schwalbach, Germany). Cells were lysed on ice by addition of 1% Triton X 100 containing lysis buffer, supplemented with 1mM 1,4-Dithiol-DL-threitol (Fluka Chemie GmbH, Buchs, Germany), Phosphatase-Inhibitor (Sigma, Steinheim, Germany) and Protease-Inhibitor (Roche, Mannheim, Germany). Cell lysates were centrifuged for 10 minutes (14.000g at 4°C) to remove insoluble debris.

### *Generation of RYSK173 CN-1 monoclonal antibody*

Recombinant human CN-1 was produced by using a cell-free rapid translation system (Roche, Mannheim, Germany) according to the manufacture's recommendations. Purified adjuvans-free recombinant human CN-1 was injected intraperitoneally into Balb/c mice to generate the RYSK173 monoclonal antibody. The mice were boosted 2 and 4 weeks after immunisation. Serum was collected 3 days after the last boost to assess CN-1 antibody titre by indirect immune fluorescence (IIF) on CN-1 transfected Cos-7 cells. Mice were sacrificed and splenocytes were fused with SP2/0 myeloma cells according to standard procedures. After fusion the cells were plated at a density of  $1 \times 10^3$  cells/well. Wells containing proliferating cells were tested for the presence of anti-carnosinase antibody by IIF. Positive cultures were subsequently seeded at a density of 70-80 cells/96 well plate and retested by IIF. Clone RYSK173 (IgG1) was selected for further analysis.

### *CN-1 ELISA*

A human CN-1 ELISA was developed by coating high absorbent microtitre plates (Greiner, Labortechnik, Frickenhausen, Germany) overnight with 100 µl of goat polyclonal anti-human

CN-1 (10 µg/ml) (R&D, Wiesbaden Germany). The Pates were extensively washed and incubated with 0.05 % W/V of dry milk powder to avoid unspecific binding. For each sample and standard serial dilution were carried out. The pates were placed on a shaker for 1 hr and subsequently extensively washed with PBS/Tween. Hereafter purified anti-human carnosinase monoclonal IgG (clone RYSK173) or rabbit polyclonal IgG (ATLAS, Abcam plc, Cambridge, United Kingdom) were added for 1 hr followed by extensively washing. Biotinylated goat anti-mouse - or goat anti-rabbit IgG was added for 1 hr, extensively washed followed by addition avidin-HRP. Deep-Blue peroxidase (POD) (Roche diagnostics, Mannheim, Germany) was used for color development, which was generally stopped after 15 minutes by addition of 50 µl of 1 M H<sub>2</sub>SO<sub>4</sub>. The plates were directly read at 450 nm. Recombinant human CN-1 (R&D Systems, Minneapolis, USA) was used as standard. CN-1 protein concentrations were assessed in the linear part of the dilution curve. Sensitivity of the ELISA assays was approximately 20 ng/ml.

### *Construction of CNDP1 mutants*

A cDNA clone of the CNDP1 gene containing 6 CTG-repeats (RZPD Library 983, entry No BX094414), kindly provided by Dr. M. Moeller (Dept. of Nephrology, RWTH, Aachen, Germany), was used as template to construct different CNDP1 variants by PCR. Glycosylation sites of CN-1 were step wise deleted using the QuikChange® II Site directed mutagenesis kit (Stratagene Europe, Amsterdam, Netherlands). In brief, single amino acid exchanges, i.e. asparagine for glycine, were induced in the wild type CNDP1 cDNA by mutagenic primers to delete N322, N382 or N402 as described by Riedl et al. (171). PCR reactions were performed according to standard procedures. All CNDP1 constructs and mutants were cloned into pCSII + mt vector. For the N-glycosylation lacking mutants, stopcodons were removed to generate a myc-tagged CN-1 protein.

### *Western blot analysis*

For detection of carnosinase in cell lysates or serum, gel electrophoreses and subsequent western blotting was performed. In some experiments samples were deglycosylated by PNGase F (New England Biolabs, Frankfurt, Germany) treatment according to the manufacturer's recommendations. All samples were boiled for 10 minutes in Laemmli sample buffer (Bio-Rad, München, Germany) prior to loading on an 8% SDS-PAGE. Proteins were transferred electrophoretically to a PVDF membrane (Roche, Mannheim, Germany) by semi-dry blotting. Hereafter the membranes were blocked for 1 hour at room temperature in TBS-

Tween 20 (0.3%, Sigma, Steinheim, Germany) containing 10% milk powder. For detection of CN-1 in transfected cells or in human serum samples, mouse monoclonal anti-CN-1 antibody (Clone RYSK-173) was utilized. After incubation with appropriate horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) antibody binding was visualized by enhanced chemiluminescence (PerkinElmer, Boston, USA).

#### *Isoelectric focusing*

Isoelectric focusing (IEF) was performed on precast 18 cm IPG strips with linear pH ranges using an IPGphor unit (Amersham Pharmacia, Freiburg, Germany). The strips were placed in ceramic strip holders and rehydrated in 350  $\mu$ L rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10) containing 10  $\mu$ g of serum proteins or 0,1  $\mu$ g of recombinant CN-1, protease inhibitors and a few grains of bromophenol blue. The strips were covered with 2 mL dry strip cover fluid (Amersham Pharmacia, Freiburg, Germany) and rehydrated over night by applying 30 V at constant temperature (20 °C) on the IPGphor unit. The following IEF protocol was used: 30 min gradient up to 500 V, 30 min 500 V, 30 min gradient up to 2000 V, 30 min 2000 V, 1 h gradient up to 8000 V, 4 h 8000 V. After focusing the strips were washed with water and immediately used for the second dimension. Before loading on SDS-polyacrylamide gels (PAGE), the strips were equilibrated for 20 min in equilibration buffer containing iodoacetamide. Hereafter the strips were carefully washed with water and transferred to the SDS-PAGE. The gels were sealed on top with agarose sealing solution (0.5% low melting agarose in 25 mM Tris, 192 mM glycine and 0.1% SDS). Electrophoresis was carried out over night in 25 mM Tris, 192 mM glycine and 0.1% SDS at 15 °C, applying a constant voltage of 100 V.

#### *Measurement of carnosinase activity:*

Serum carnosinase activity (CN-1) was assayed according to a method described by Teufel et al. (55). Shortly, the reaction was initiated by addition of carnosine and/or homocarnosine and was stopped by adding 1% trichloroacetic acid. Liberated histidine was derivatized by adding o-phthalaldehyde (OPA) and fluorescence was read using a MicroTek plate reader ( $\lambda$ Exc: 360 nm;  $\lambda$ Em: 460 nm).

#### *CNDP1 genotyping*

Genomic DNA and CNDP1 genotyping was performed as described previously [16].



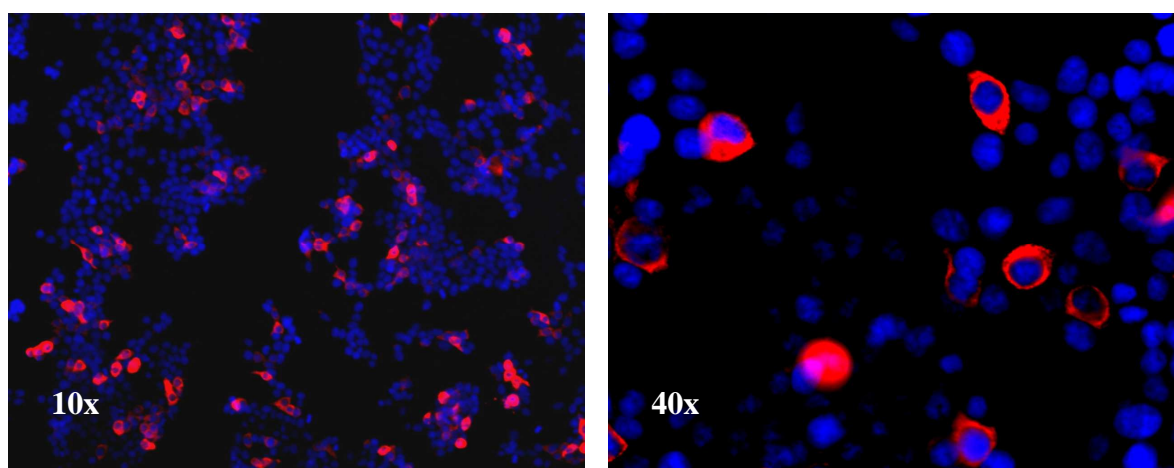
### *Statistical analysis*

Quantitative data are given as mean  $\pm$  SD. Student t-test was calculated to compare groups. Values of  $p < 0.05$  were considered as statistically significant.

## RESULTS

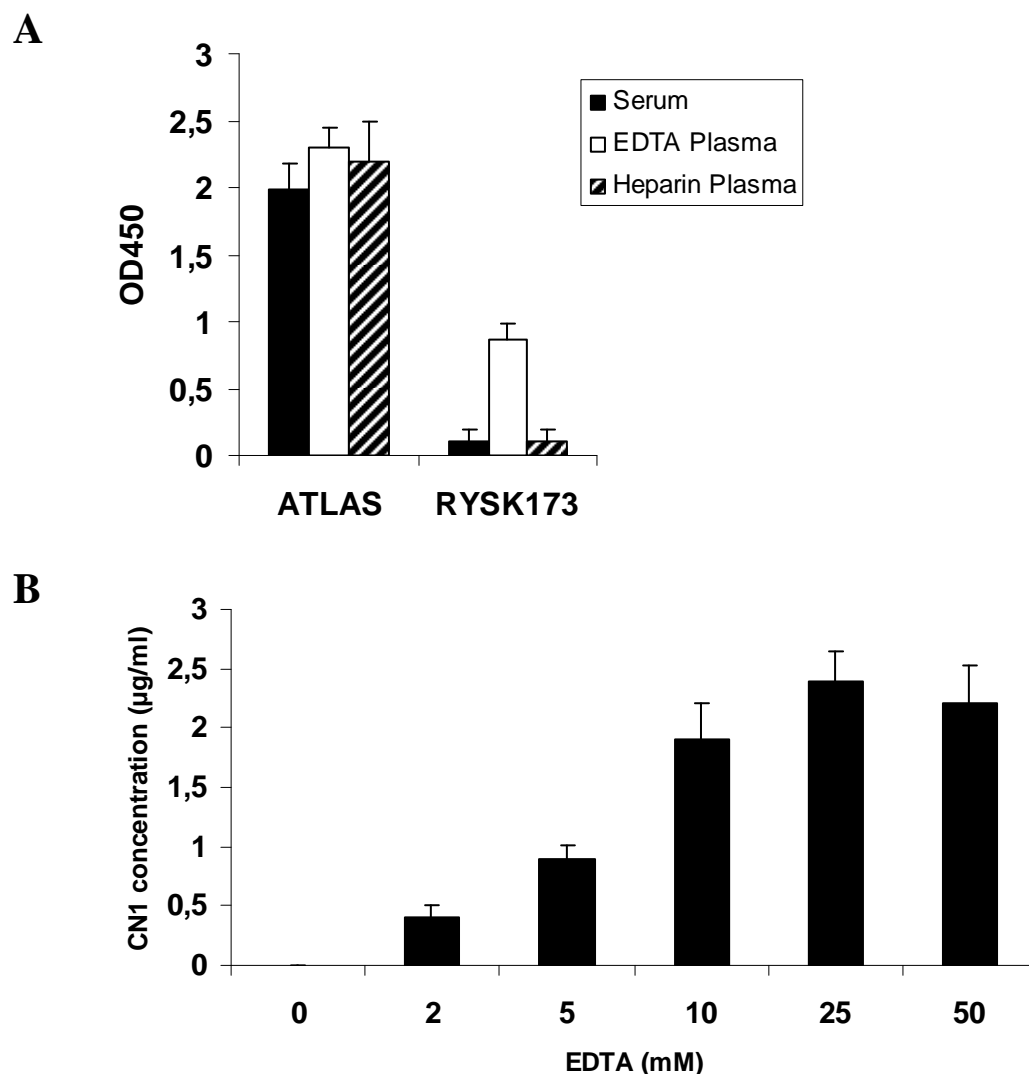
Mouse splenocytes were fused with SP2/0 myeloma cells and plated at a density of  $1 \times 10^3$  cells/well. A total of 740 supernatants obtained from the cells 2 to 3 weeks after fusion were screened for CN-1 binding by means of indirect immune-fluorescence (IIF) on cytopspins made from CN-1 transfected Cos-7 cells. Out of the initial screen 12 supernatants were found to be moderate positive in the IIF. These cells were further expanded and sub-cloned by limiting dilution at a concentration of 70-80 cells/96 well plate cells/well. Based on the strong positive staining in the IIF, one clone (RYSK173) was selected after sub-cloning for further analysis (Fig 1).

**FIGURE 1**



*Fig. 1. Expression of CN-1 in transfected Cos-7 cells. Three days after transfection cover slips of CN-1 transfected Cos-7 cells were incubated with hybridoma supernatants. Shown is the detection of CN-1 by RYSK173.*

In addition to RYSK173, a rabbit polyclonal IgG (ATLAS) was used to set-up ELISA assays for detection of CN-1 in serum and EDTA or heparin plasma. CN-1 detection by RYSK173 clearly differed, both qualitatively and quantitatively, from CN-1 detection by ATLAS. In the latter no differences were found for CN-1 detection in serum and EDTA or heparin plasma, while in the former detection of CN-1 was low in serum or heparin plasma but was significantly increased when EDTA plasma was used (Fig 2A). Although addition of EDTA to serum dose-dependently increased the detection of CN-1 with the RYSK-based assay (Fig 2B), it was quantitatively significant lower compared with the ATLAS-based assay (RYSK173 vs. ATLAS,  $2.43 \pm 0.52$  vs.  $22.5 \pm 3.8$   $\mu\text{g/ml}$ ;  $P < 0.01$ ).

**FIGURE 2**

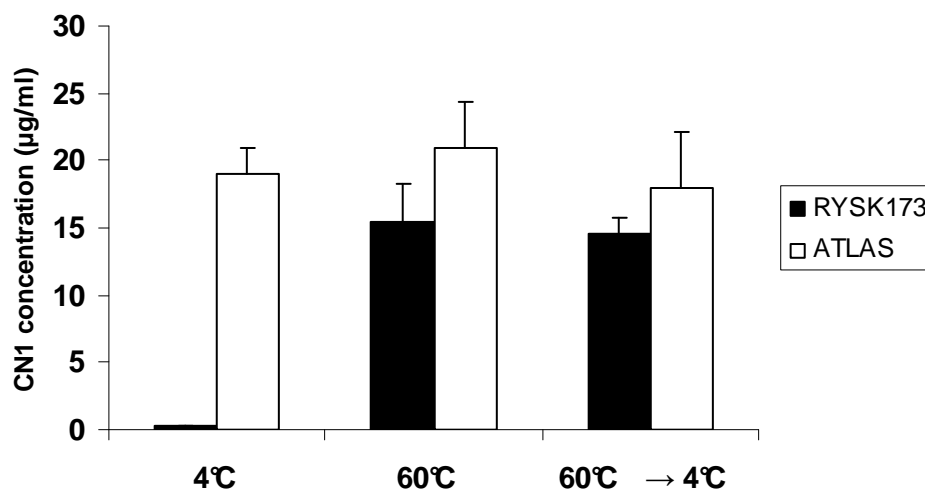
*Fig. 2. Performance of CN-1 ELISA assays in serum and plasma samples. A: Serum, EDTA - and heparin plasma obtained from the same individual were tested in ELISA for CN-1 using ATLAS or RYSK173 as detecting antibody. The results of a representative experiment is shown and expressed as mean OD450 value  $\pm$  SD. B: Different concentrations of EDTA were added to a serum sample and CN-1 was subsequently assessed in ELISA by RYSK173. In each assay a standard curve with recombinant C N-1 was included to assess the concentration of CN-1 in the samples. The results are obtained from one individual and expressed as mean concentration ( $\mu\text{g/ml}$ )  $\pm$  SD of triplicate measurements. A total of 4 different individuals were tested, with essentially similar results.*

Apart from the addition of EDTA, detection of CN-1 with the RYSK-based assay could also be increased by protein denaturation. This was done either by incubating serum for 30 minutes at 60°C or by addition of the reducing agent DTT (Fig. 3). Detection of CN-1 with the RYSK-based assay dramatically increased when serum was incubated at 60°C, and

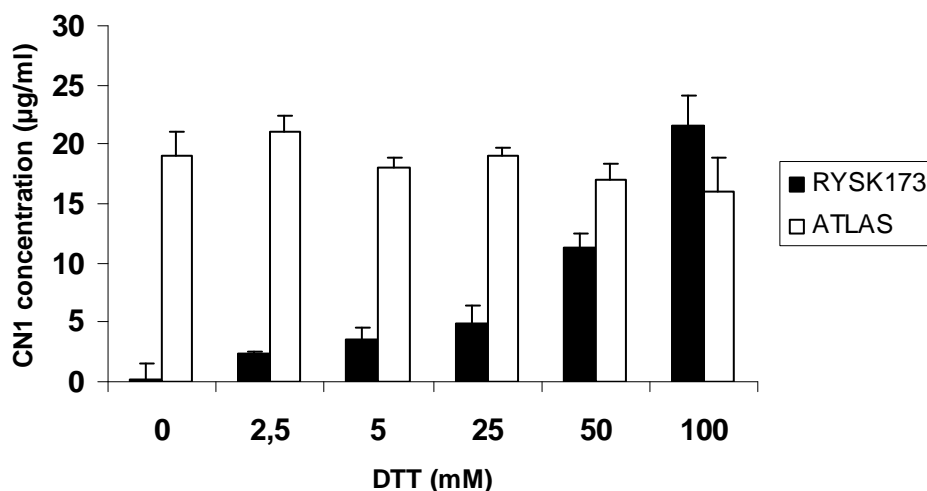
remained high when the heated serum was placed at 4°C for an additional 20 minutes (Fig 3A). Similarly, addition of DTT dose-dependently increased the detection of CN-1 with the RYSK-based assay to the level as observed for the ATLAS-based assay (Fig. 3B).

**FIGURE 3**

**A**



**B**



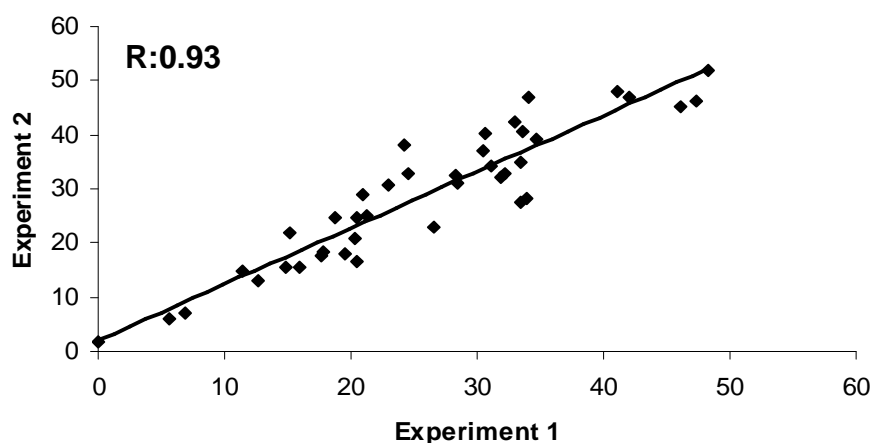
*Fig. 3. CN-1 detection by RYSK173 is increased upon denaturation of serum. A: Serum was either incubated at 4°C or 60°C for 30 minutes. In addition, a serum sample was first incubated at 60°C for 30 minutes and subsequently put on ice for 20 minutes before assessment of CN-1. In all samples CN-1 was detected by ELISA using ATLAS or RYK173 as detecting antibody. B: Different concentrations of DTT were added to serum. Hereafter CN-1 was detected by ELISA using ATLAS or RYK173 as detecting antibody. In A and B the results of a representative experiment are depicted and expressed as mean concentration (µg/ml) ± SD of triplicate measurements. A total of 4 different individuals were tested, with essentially similar results.*

Reproducibility of both ELISA assays was assessed by consecutive measurements of 40 serum samples on different days. As indicated in figure 4 both ELISA's were highly reproducible with a correlation coefficient (R) of 0.93 ( $P<0.01$ ) and 0.99 ( $P<0.01$ ) for the ATLAS- and RYSK-based assays respectively (Fig 4A and B).

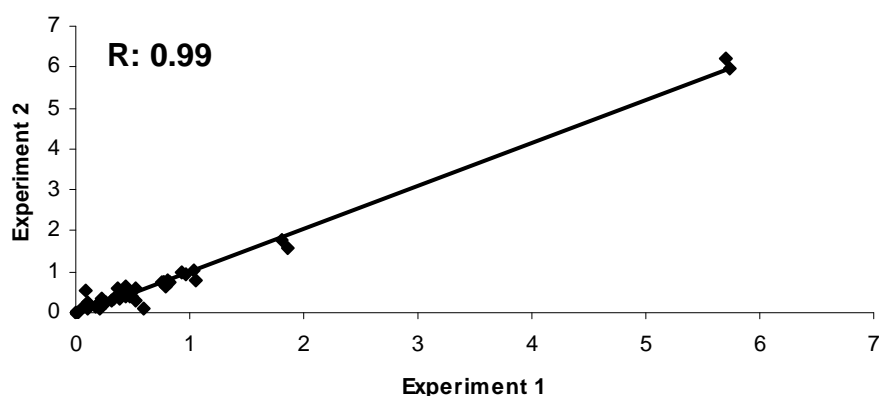
When the relative contribution of CN-1 detected with the RYSK-based assay to total CN-1 detected with the ATLAS-based assay was calculated, there was also a good reproducibility observed. CN-1 detected by the RYSK-based assay was in most samples below 5 % of total CN-1. However in 7 out of 40 samples this was clearly higher (Fig 4C).

**FIGURE 4**

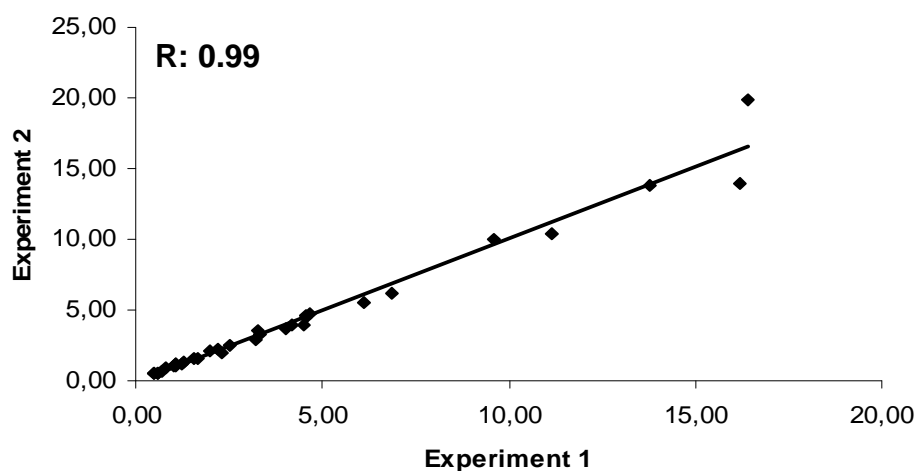
**A**



**B**



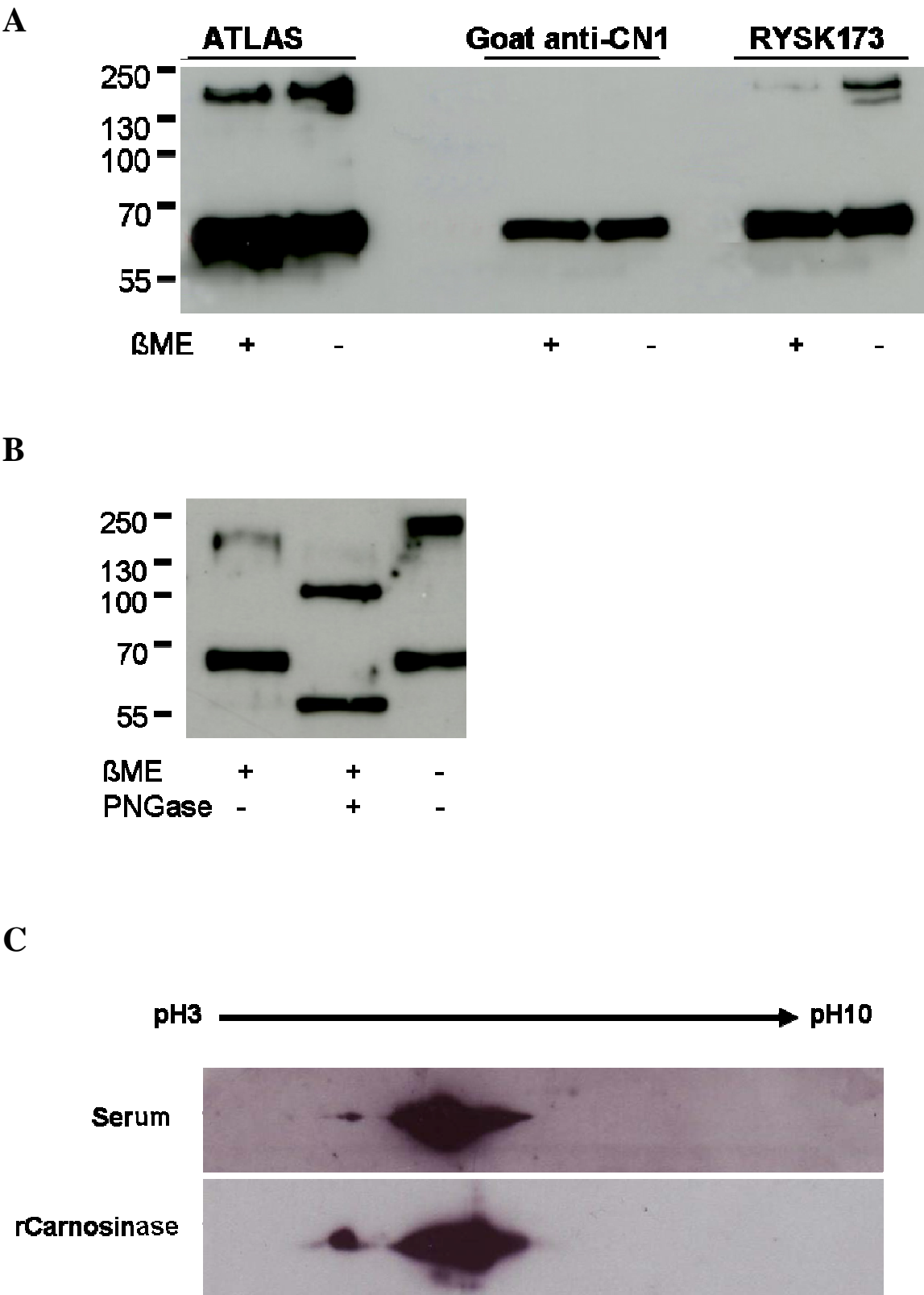
C

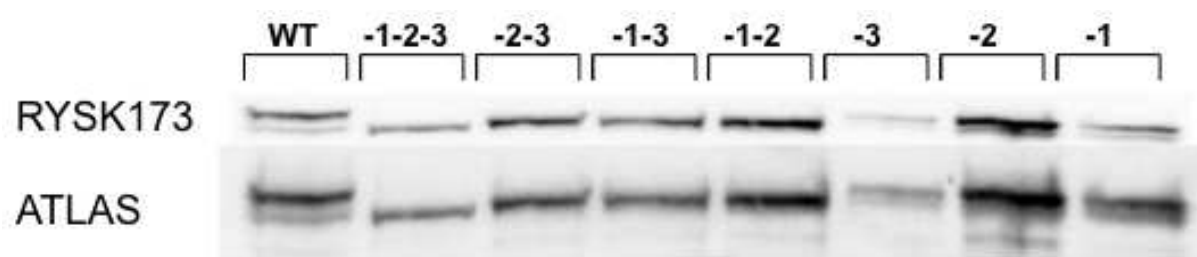


*Fig. 4. Reproducibility of CN-1 detection in the ATLAS-based (A) and the RYSK-based (B) assay. A total of 40 serum samples were measured on two different days. C: the relative contribution of CN-1 detected by the RYSK-based assay to total CN-1 was calculated as follows: (CN-1 measured with the RYSK-based assay/ CN-1 measured with the ATLAS-based assay) x 100 %*

To investigate whether RYSK173 was detecting a different molecular weight variant of CN-1, we performed western blot analyses of serum samples with all anti-CN-1 antibodies that were used in ELISA, including the catching goat anti-human CN-1 polyclonal IgG. As depicted in figure 5A, all anti-CN-1 antibodies recognized a 65 kDa band under reducing and non reducing conditions. While ATLAS, and to a lesser extent RYSK173, also reacted with a high molecular weight band of approximately 150 kDa, this was not observed with the goat-anti-human CN-1 polyclonal IgG from R&D. To assess whether the upper band was a differentially N-glycosylated CN-1 isoform, aliquots of the samples were treated with PNGase. PNGase treatment resulted in a shift of both bands as shown in figure 5B. Isoelectric focusing of human serum and recombinant CN-1 followed by PAGE and western blotting with RYSK173 showed that RYSK173 was recognizing a major protein with the expected pI of 4.4 for serum carnosinase (Fig 5C). Because not all putative N-glycosylation sites within CN-1 are necessarily used for glycosylation, we also tested if RYSK173 was recognizing differentially N-glycosylated CN-1 forms. To this end, we transfected Cos-7 cells with myc-tagged CN-1 variants that lack 1, 2 or all 3 putative N-glycosylation sites by exchanging asparagine residues at N322, N382 and N402 for glycine. Although the expression of the differentially glycosylated variants largely differ, all variants were recognized by RYSK173 and ATLAS to a similar extent (Fig 5D).

FIGURE 5



**D**

*Fig. 5. Detection of CN-1 in serum by western blot analysis. A: Serum was subjected to PAGE under reducing (+) or non-reducing (-) conditions. Reducing conditions were achieved by the addition of  $\beta$ -mercaptoethanol ( $\beta$ ME) to the sample buffer. Western blotting was performed using ATLAS (rabbit polyclonal anti-CN-1 IgG), Goat polyclonal IgG and RYSK173 (mouse monoclonal anti-CN-1 IgG1). B: To assess if the different bands at 150 and 65 kDa respectively were different N-glycosylated CN-1 variants serum samples were treated with PNGase (+) or not (-). Similar as in A, PAGE was performed under reducing or non-reducing conditions by adding  $\beta$ ME (+) or not (-). C: Serum and recombinant CN-1 were subjected to 2D-gel electrophoresis as described in materials and methods. After the second dimension the gel was blotted and incubated with RYSK173 for detection of CN-1. D: Cos-7 cells were transfected with different CN-1 cDNAs lacking 1, 2 or all 3 N-glycosylation sites. Mutations in N-glycosylation sites at position 1 (N322), position 2 (N382) and position 3 (N402) or combinations hereof are depicted by (-) followed by the position number. Cell extracts of transfected cells were subjected to PAGE and western blotting using RYSK173. Hereafter the blot was stripped and re-probed with ATLAS.*

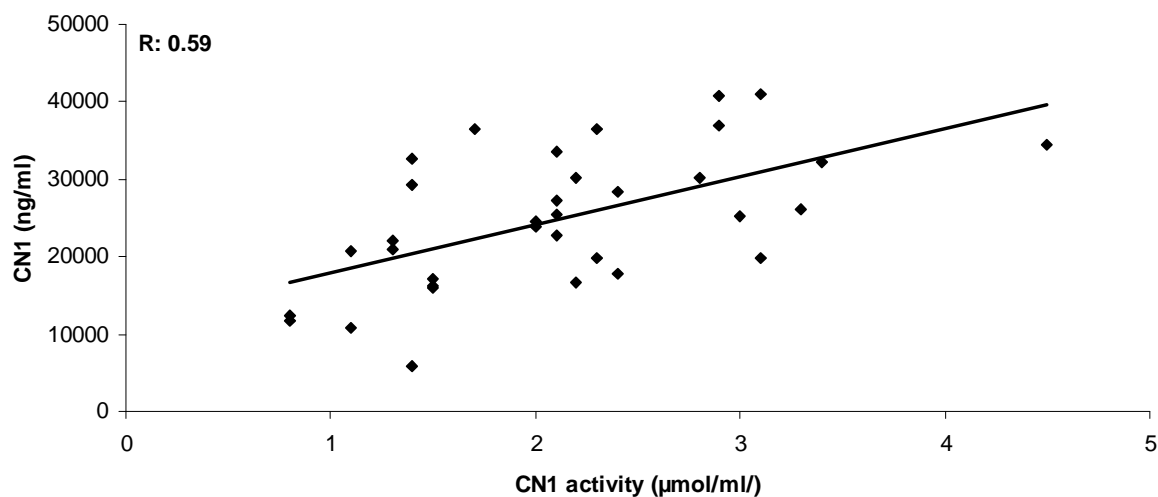
We next assessed in a total of 33 serum and corresponding EDTA samples CN-1 concentrations using both ELISA assays and addressed whether enzyme activity correlated with serum CN-1 concentrations. As already shown in figure 2, the amount of CN-1 detected with the ATLAS-based assay did not significantly differ between serum and EDTA plasma (for serum: mean  $\pm$  SD  $24.8 \pm 8.9$  [range 11.7 – 40.8]  $\mu$ g/ml, for plasma  $17.6 \pm 5.3$  [range 9.3 – 27.2]). While in most sera the concentration of CN-1 detected with the RYSK-based assay was low (mean  $\pm$  SD  $0.2 \pm 0.8$  [range 0 – 4,8]  $\mu$ g/ml), for all individuals CN-1 concentrations were significantly higher in EDTA plasma when tested in the RYSK-based assay. These concentrations were however significantly lower when compared to ATLAS-based assay (mean  $\pm$  SD  $2 \pm 2.1$  [range 0.4 -11.6]  $\mu$ g/ml,  $P < 0.01$ ). CN-1 concentrations assessed by the latter assay correlated with CN-1 activity ( $R: 0.59$ ,  $P < 0.01$ , Fig 6A), but this was not found for the RYSK-based assay (data not shown). However, there was a negative correlation between CN-1 activity and the proportion of CN-1 detected with the RYSK-based assay, suggesting that the RYSK-based assay most likely detects a CN-1 conformation with low activity ( $R: -0.51$ ,  $P < 0.01$ , Fig 6B). Similar as reported previously for CN-1 activity, it was



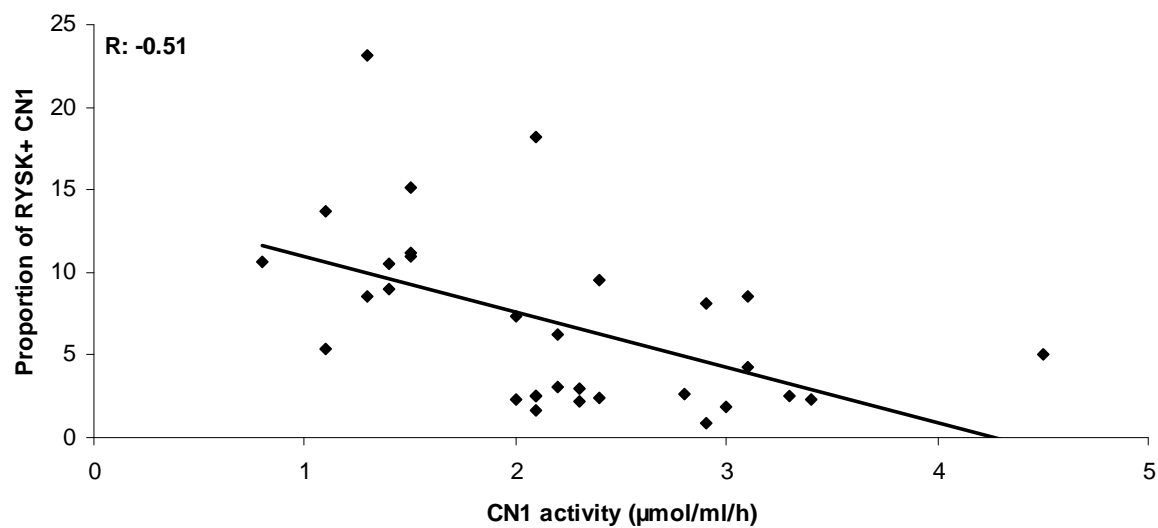
found that in (CTG)<sub>5</sub> homozygous individuals CN-1 concentrations were significant lower compared to other (CTG)<sub>n</sub> genotypes (Fig 6C).

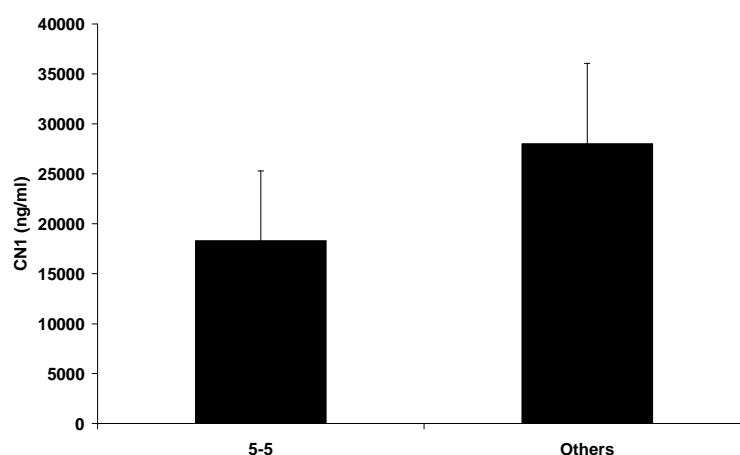
**FIGURE 6**

**A**



**B**



**C**

*Fig. 6. Detection of CN-1 in a cohort of different individuals. A: Correlation between CN-1 protein concentrations and CN-1 activity in serum. CN-1 protein concentrations were assessed by ELISA using ATLAS. B: Correlation between CN-1 activity and the proportion of CN-1 detected in the RYSK-based assay. The proportion of CN-1 detected by the RYSK-based assay (RYSK+ CN-1) to total CN-1 was calculated as follows: (CN-1 measured with the RYSK-based assay/ CN-1 measured with the ATLAS-based assay) x 100 %. C: CN-1 protein concentrations in serum of homozygous (CTG)<sub>5</sub> individuals (5-5) and in individuals carrying a different genotype (others)*

**DISCUSSION**

To date, most available methods for measuring carnosinase in serum are based on measuring enzyme activity (54, 59, 172). CN-1 activity is, however, modulated by binding of metal ions (54), the presence of homocarnosine (169), and by N-glycosylation of the enzyme (170). Hence, carnosinase activity might be discordant to carnosinase protein concentrations. To assess whether low carnosinase activity is due to low enzyme concentrations it is therefore crucial to estimate the concentration of CN-1. In the present study we describe ELISA assays for assessment of CN-1 in serum or plasma samples. The main findings of this study are the following. Firstly, CN-1 concentrations can be assessed by ELISA in a highly reproducible manner. While the ATLAS-based assay correlates with CN-1 activity, this was not found for the RYSK-based assay. Secondly, CN-1 seems to be present in different conformations as only a fraction of CN-1 detected with the ATLAS-based assay is also detected with the RYSK-based assay. Thirdly, detection of CN-1 with the RYSK-based assay is increased by addition of EDTA to serum or by denaturation of serum proteins.

Because ATLAS and RYSK173 showed marked differences in ELISA it could be argued that these antibodies recognize structurally different CN-1 variants. This is however unlikely since in western blot analysis similar bands were recognized by both antibodies. Moreover, in 2D-PAGE and subsequent western blotting RYSK173 recognized one major spot with comparable pI of 4.4 as reported for CN-1 (55). CN-1 was recognized by RYSK173 both in its N-glycosylated and deglycosylated form as demonstrated by PNGase treatment. Preferential recognition of a differentially N-glycosylated form of CN-1 by RYSK173 is also unlikely since both RYSK173 and ATLAS gave comparable results when all possible combinations of N-glycosylation CN-1 mutants were tested in western blotting.

It is more likely that recognition of CN-1 by RYSK173 in ELISA is dependent on the CN-1 conformation. Recently, a study by Vistoli et al (173) has postulated that citrate ions can alter the conformation and activity of CN-1. Given the fact that in the active centre of CN-1 different metal ions may be bound, it is conceivable that this might result in different CN-1 conformations. Qualitative differences in - or the lack of - metal ion binding might explain why CN-1 concentration detected with the RYSK-based assay are generally low and why this varies between individuals that do have comparable amounts of CN-1 detected with the ATLAS-based assay. This notion is supported by the fact that addition of EDTA increased detection of CN-1, albeit that the amount of CN-1 that was detected was still significantly lower to that detected by ATLAS. Based on a differential affinity of metal ions for EDTA, it

is conceivable that not all metal ions within CN-1 can be chelated by EDTA. Consequently, only metal ions with a high affinity for EDTA will be chelated. If metal ion binding is masking the RYSK173 epitope, this explains why only a proportion of CN-1 can be detected when EDTA is added to serum samples. Since RYSK173 is also recognizing CN-1 under denaturing conditions, our data strongly suggest that RYSK173 is not recognizing a conformational epitope but more likely a linear epitope that is masked in a varying number of CN-1 molecules. We can not exclude however, that detection of CN-1 in serum with the RYSK-based assay is caused by damage or denaturation of the native CN-1 protein.

Previously, we have shown that the polymorphic (CTG)<sub>5</sub> repeat in the *CNDPI* gene is a susceptibility locus for developing DN (44). We have also shown that in individuals homozygous for the (CTG)<sub>5</sub> allele, CN-1 activity is low (44) and that this might be due to a poor secretion of CN-1 in individuals carrying this genotype (168). We now not only show that CN-1 activity correlates with CN-1 protein concentrations when assessed by ATLAS, but also that indeed (CTG)<sub>5</sub> homozygous individuals have significant lower CN-1 concentrations compared to individuals with other genotypes. Although CN-1 concentrations assessed with the RYSK-based assay did not correlate with CN-1 activity, CN-1 activity inversely correlated with the proportion of CN-1 that was recognized by RYSK173. This therefore suggests that RYSK173 might recognize a CN-1 conformation with low enzyme activity. Both explanations for detection of CN-1 in serum with the RYSK-based assay, i.e. differential metal ion binding (173) or denaturation of CN-1, fit with the assumption that RYSK173 recognizes a CN-1 conformation with low enzyme activity.

In conclusion, we have shown that CN-1 concentrations can be detected by ELISA in a highly reproducible manner. We have developed two ELISA assays, which can be used for quantitative and qualitative CN-1 measurements. While the ATLAS-based assay demonstrates a good correlation between enzyme activity and CN-1 protein concentration, the RYSK-based assay seems to detect a specific CN-1 conformation. Although we cannot discriminate whether this conformation is related to differential metal ion binding or damage of the native CN-1 protein, it seems that RYSK173 is recognizing a CN-1 variant with low enzyme activity. The relevance of these different CN-1 conformations is still elusive and needs to be addressed in further studies.

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